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The underlying mechanism of the progression of prostate cancer to hormone-independent disease is poorly understood. Neexpression of caveolin-1, a scaffold protein associated with caveolae membrane microdomains, has been shown to correlate with hormone resistance and metastasis in both human and mouse prostate cancer models. We found overexpressing caveolin-1 in human prostate cancer cells postively regulate androgen receptor transactivation activity. We identify in cellular models that modulating caveolin expressing levels dramatically alter the sensitivity of AR to androgen stimulation. We hypothesize that caveolin-1 sacffoding signal complex play a regulatory role in AR activation pathway. The immediate goal of the present proposal aims to identify and characterize the sub-moleclar domains involved in AR/caveolin interaction. In current funding year, we demonstrated a cross-talk between the caveolin-1-associated signal pathway and AR-mediated transcriptional activity in our first funding year. Several lines of evidence, biological as well as biochemical, support the notion of an androgen-dependent physiological interaction between these two pathways. Overall, our results suggest that caveolin-1 plays a role as a convergent point for AR cross-talk with other cellular signal transduction pathways. These findings pave the way to further define the underlying signal cross-talk in AR-mediated transactivation.

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## **DOD Progress Report:**

### **Caveolin-1 Modulates Androgen Receptor Signaling in Advanced Prostate Cancer**

#### **Introduction:**

The underlying mechanism of the progression of prostate cancer to hormone-independent disease is poorly understood. Neoeexpression of caveolin-1, a scaffold protein associated with caveolae membrane microdomains, has been shown to correlate with hormone resistance and metastasis in both human and mouse prostate cancer models (Nasu et al., 1998; Yang et al., 1999). We find that overexpressing caveolin-1 in human prostate cancer cells positively regulates androgen receptor transactivation activity. We identify, in cellular models, that modulating caveolin expressing levels dramatically alter the sensitivity of AR to androgen stimulation (Lu et al., 2002). We hypothesize that caveolin-1 scaffolding signal complex plays a regulatory role in AR activation pathway. Our specific aims are: (1) Mapping the submolecular domains required for AR and caveolin interaction; (2) Functional and biochemical characterization of the AR/caveolin interaction; (3) Characterization of the physiological role of caveolin-1 overexpression in AR signaling of prostate carcinoma cell; (4) Evaluating the effect of caveolin scaffolding domain CSD peptide in prostate cancer PC3 tumor growth in vivo. The immediate goal of the present proposal aims to identify and characterize the sub-molecular domains involved in AR/caveolin interaction.

#### **Report Body:**

In our current funding year, we have completed the majority part of the Task1 which includes the construction of domain specific deletion mutants of both androgen receptor and caveolin-1 fused to mammalian two-hybrid interaction vectors pVP and pGal by molecular cloning. The resulting constructs were fully sequenced to verify the correct nucleotide sequences. The correct expression of these fusion proteins was also verified by western blot analysis using cell lysates derived from expression vector transfected PC3 or HeLa cells. Based on the results from mammalian two-hybrid assays, we have determined two caveolin interacting domains in androgen receptor (AR) which localized to AR N-terminus and ligand binding domain (LBD). The AR binding domain of caveolin was determined to localize to the first 60 amino acids of caveolin.

To biochemically verify the above-mentioned interacting domains, we also constructed the expression vector that directs the expression of GST-fusion proteins with various domains derived from either AR or caveolin. Biochemical interactions as determined by GST-pull down and co-immunoprecipitation experiments provided further evidence for the AR caveolin interaction.

Based on the mapping data described above, we started testing the hypothesis that peptide and peptide domains identified from Task 1 derived from caveolin scaffolding domains (CSD) may modulate the growth regulation of PC3 cells. In this respect, we have generated expression vectors for the specific peptides including CSD, Cav(1-60) and Cav(137-178) for testing their effects using PC3 cells in a tissue culture model.

#### Key Research Accomplishments:

1. Identification of AR sub-molecular domains, AR-N and LBD, interacting with caveolin
2. Identification of caveolin sub-molecular domain, CSD, interacting with AR
3. Established the physiological interactions between AR and caveolin-1 at both molecular and biochemical levels.

#### Reportable Outcomes:

1. Manuscript:  
Zhuang L., Lin J., Lu M.L., Solomon K.R. Freeman M.R., (2002)  
Cholesterol-rich Lipid Rafts mediate Akt-regulated Survival in Prostate Cancer Cells. *Cancer Research* 62: 2227-2232. (appendix)
2. Cell Line:  
LNCap cell stably expressing caveolin, LNCav
3. Expression Vectors:  
Mammalian expression vectors for AR, caveolin expression. pcDNA-AR. pcDNA-Cav, pVp-AR and pGal-Cav

#### Conclusion:

In summary, we have demonstrated a cross-talk between the caveolin-1-associated signal pathway and AR-mediated transcriptional activity in our first funding year. Several lines of evidence, biological as well as biochemical, support the notion of

an androgen-dependent physiological interaction between these two pathways. Overall, our results favor the notion that a transient and dynamic association between AR and caveolin-1 may play a role in promoting AR ligand-dependent transcriptional activation. Although the functional significance of this interaction remains to be determined, it suggests that caveolin-1 plays a role as a convergent point for AR cross-talk with other cellular signal transduction pathways. These findings pave the way to further define the underlying signal cross-talk in AR-mediated transactivation.

#### References:

1. Lu M.L., Schneider M.C., Xheng Y., Zhang X. and Richie J.P. J. Biol.Chem. **276**, 13442 (2001).
2. Nasu Y., Timme T. L., Yang G et al., Nature Med. **4**, 1062 (1998).
3. Yang G., Trong L.D. Wheeler T.M. and Thompson T.C. Cancer Res. **59**, 5719 (1999).

# Cholesterol-rich Lipid Rafts Mediate Akt-regulated Survival in Prostate Cancer Cells<sup>1</sup>

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## Abstract

Although cholesterol accumulation in tumors was first reported in the early 20th century, the mechanistic implications of this observation are still obscure. Here we report that caveolin-negative human prostate cancer (LNCaP) cells contain cholesterol-rich lipid rafts that mediate epidermal growth factor (EGF)-induced and constitutive signaling through the Akt1 serine-threonine kinase. EGF receptor and Akt1 phosphorylation were inhibited and autonomous cell survival was reduced when the rafts were disrupted. Reconstitution of the rafts with cholesterol restored EGF receptor→Akt1 axis signaling and cytoprotection from a phosphoinositide 3-kinase-dependent apoptotic signal. These results suggest that cholesterol present in membrane microdomains is a prominent mediator of survival in prostate cancer cells.

## Introduction

PCa<sup>5</sup> incidence and progression have been linked to high-fat "Western" diets, including intake of animal products (1, 2). The mechanism by which PCa cell growth and/or cell survival might be promoted by dietary fat is poorly understood. White (3) reported in 1909 and subsequent studies have also demonstrated that solid tumors (4, 5), including those of the prostate (6), accumulate cholesterol and other fatty deposits. Several older reports have suggested an association between malignancy and cholesterol levels specifically in prostatic tissue and secretions (6, 7). More recently, caveolin-1, the main structural protein of caveolar lipid membrane microdomains, generally known as lipid rafts (8), was identified as a protein that may be functionally associated with tumor progression and metastasis in PCa (9-11). Caveolae and other types of lipid rafts are now known to serve as membrane platforms for cell signaling (12, 13). Importantly, lipid raft microdomains are enriched in cholesterol relative to the liquid-disordered component of the cell membrane, suggesting a role for membrane cholesterol as a determinant of growth factor-mediated cell survival mechanisms.

To test the hypothesis that membrane cholesterol plays a role in cell survival in PCa cells, we used LNCaP human PCa cells that do not express caveolins. The absence of these proteins, which sequester signaling molecules, eliminates the possibility of functional contributions from caveolin-associated membrane complexes. LNCaP cells are also useful as a model system because they express prostate-

specific properties and are dependent for cell survival on a constitutive signal through the PI3K/Akt phosphorylation cascade (14). The essential role of this signaling mechanism in this cell line may be related to the presence of an inactivating mutation in the gene encoding the PTEN phosphatase, a negative regulator of this pathway. Loss of PTEN is a frequent event in human PCa (15, 16), and up-regulation of the PI3K/Akt pathway has been linked to PCa progression (17).

## Materials and Methods

**Cell Culture.** Human PCa cell lines LNCaP and PC-3 were purchased from the American Type Culture Collection (Rockville, MD). Both cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS. LNCaP cells transfected with the plasmid pcDNA-Cav-1 or with an empty vector were cultured in medium containing 300 µg/ml G418 as described (18). Details of specific cell treatments are described in the figure legends.

**Antibodies and Reagents.** The following mAbs and pAbs were used: anti-EGFR pAb (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phosphorylated EGFR mAb, anti-Giα3 pAb (Calbiochem, La Jolla, CA); anti-caveolin-1 mAb (clone 2297), anti-caveolin pAb, anti-Fyn mAb (clone 25; Transduction Labs, San Diego, CA); anti-Akt pAb, anti-phosphorylated Akt pAb (Cell Signaling, Beverly, MA). Human recombinant EGF and HB-EGF were purchased from R&D (Minneapolis, MN). Filipin, cholesterol, and cyclodextrin were from Sigma Chemical Co. (St. Louis, MO).

**Successive Detergent Extraction of Lipid Rafts.** Extraction of Triton-soluble and -insoluble membrane constituents was performed essentially as described (19). In brief, cells were resuspended in buffer A [25 mM 2-(N-morpholino)ethanesulfonic acid, 150 mM NaCl, pH 6.5]. To this, an equal volume of the same buffer with 2% Triton X-100, 2 mM Na<sub>3</sub>VO<sub>4</sub>, and 2 mM phenylmethylsulfonyl fluoride was added, and the cells were incubated on ice for 30 min. Insoluble fractions were pelleted in a microcentrifuge (14,000 × g) for 20 min at 4°C. The supernatant was removed ["S" (soluble) fraction], and the insoluble pellet was resuspended in buffer B [1% Triton X-100, 10 mM Tris (pH 7.6), 500 mM NaCl, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 60 mM β-octylglucoside (Sigma Chemical Co.), and 1 mM phenylmethylsulfonyl fluoride] for 30 min on ice. Debris was pelleted in a microcentrifuge (14,000 × g) for 20 min at 4°C, and the supernatant was collected. This fraction is referred to as "I" (insoluble). Immunoblotting was performed as described (14).

**Apoptosis Assays.** A quantitative sandwich ELISA was performed to measure mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates according to the manufacturer's manual (cell death detection ELISA; Roche, Indianapolis, IN). Briefly, 1.5–2.0 × 10<sup>5</sup> cells/well were seeded in six-well plates for 24 h, cell lysates were collected after various treatments as indicated, and the amount of histone-associated DNA fragments was quantified by spectrophotometric measurement of peroxidase activity retained in the immunocomplex (415 nm) against the substrate solution as a blank (490 nm). Apoptosis was also evaluated by the TUNEL method, using the *In Situ* Cell Death Detection kit (Roche). Briefly, 1.5–2.0 × 10<sup>5</sup> cells/well were seeded in six-well plates for 24 h, and cells were collected by scraping. Cells were fixed in 4% paraformaldehyde and permeabilized, and DNA was labeled with fluorescein using the TUNEL reaction mix. The percentage of apoptotic cells was determined by flow cytometry. Apoptosis induced by filipin was confirmed by the ladder genomic DNA fragmentation assay as described (14). Briefly, cells (8.5 × 10<sup>5</sup>) seeded in 6-cm dishes for 3 days in 10% FBS RPMI 1640 were cultured in serum-free medium and subjected to various treatments

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<sup>5</sup> The abbreviations used are: PCa, prostate cancer; PI3K, phosphoinositide 3-kinase; mAb, monoclonal antibody; pAb, polyclonal antibody; EGF, epidermal growth factor; EGFR, EGF receptor; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; SDEM, successive detergent extraction method.

(see figure legends). Subsequently, the cell DNA was extracted, precipitated, separated in 1.8% agarose gels, and visualized by ethidium bromide staining. The image in Fig. 3C was generated using AlphaEase software (Alpha Inno-tech Corp., San Leandro, CA) and a PC-controlled transilluminator.

# Results and Discussion

LNCaP cells obtained from American Type Culture Collection were lysed and fractionated into Triton-soluble (S) and Triton-insoluble/octylglucoside-soluble (I) fractions. Under the lysis conditions used in these experiments, lipid raft/caveolae components partition into the I-fraction (19). A comparison between LNCaP cells and caveolin-positive PC-3 human PCa cells revealed that the Src family kinase, Fyn, and the heterotrimeric G-protein subunit  $G_{i\alpha 3}$ , both shown previously to partition into lipid raft/caveolae microdomains (13, 19, 20), were similarly distributed and enriched in the I-fraction of both cell types (Fig. 1). Caveolin-1 partitioned nearly completely (>95%) into the Triton-insoluble/octylglucoside-soluble fraction in PC-3 cells, validating that the I-fraction consisted of the components of caveolae and related lipid rafts as expected. Caveolin monomers, which migrate in the  $M_r$  21,000–24,000 range in SDS-PAGE gels, were not detected in the LNCaP cells used in these studies, consistent with some previous reports (9, 18). [LNCaP cell variants expressing caveolin-1 have also been reported (21).] These findings indicate that LNCaP cells possess a lipid raft compartment that is not dependent on the presence of caveolin-1.

EGFR-mediated activation of the PI3K/Akt signaling pathway has been shown to promote cell survival in LNCaP and other cell types, suggesting an important role for this signaling system in PCa progression (22). Because EGFR activation was demonstrated to be regulated by lipid rafts in other cell types (23), we investigated their possible biological function in regulating EGFR signaling in LNCaP cells. Serum-starved cells were treated with EGF, and levels of total and phosphorylated forms of the EGFR were examined in S- and I-fractions. EGFR was predominantly located in the S-fraction (Fig. 2). In fact, the EGFR was nearly undetectable in the I-fraction [on overexposed immunoblots a small amount of EGFR could be visualized in the I-fraction (data not shown)]. Importantly, ligand-induced phosphorylation of EGFR (on Tyr-1173) was predominantly seen in the I-fraction (Fig. 2), although EGF treatment did not appear to cause detectable redistribution of EGFR.

To further investigate the involvement of lipid rafts in EGFR signaling, LNCaP cells were treated with the raft-disrupting agent, filipin, a polyene macrolide that binds cholesterol with high specificity (24–26). Filipin has been shown repeatedly to disrupt lipid raft-dependent signaling and transport events (27–29). Filipin pretreatment (2  $\mu$ g/ml) suppressed ligand-dependent EGFR phosphorylation in the I-fraction. After reconstitution of the raft domains with cholesterol,

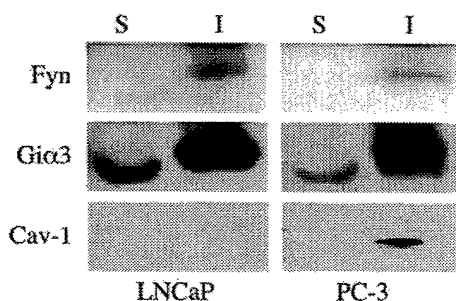


Fig. 1. Membrane lipid rafts in prostate cancer cells. LNCaP cells and PC-3 cell lysates were subjected to a SDEM, and equal amounts of protein from the Triton-soluble (S) and Triton-insoluble (I) fractions were electrophoresed on 4–20% gradient SDS-polyacrylamide gels, electrotransferred and immunoblotted with antibodies for the indicated proteins.

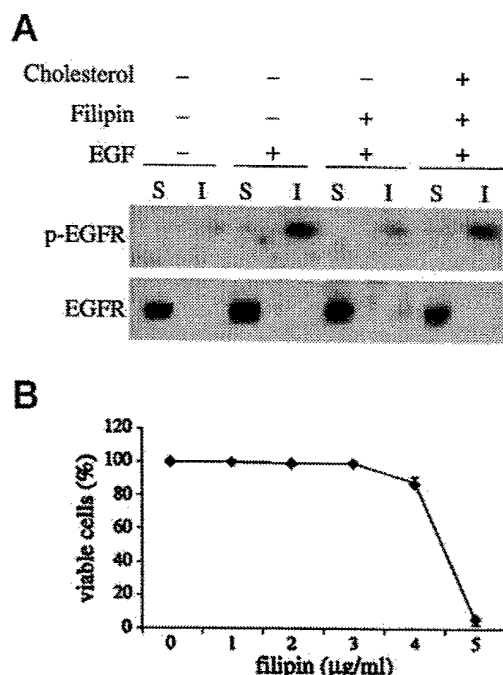


Fig. 2. EGFR signaling occurs in a lipid raft membrane compartment. LNCaP cells were seeded on poly-L-lysine-coated six-well plates overnight and starved for 16–20 h in serum-free medium. A, mock- or filipin-pretreated (2  $\mu$ g/ml) cells were incubated in serum-free medium with or without cholesterol/cyclodextrin (Cholesterol) complexes for 1 h at 37°C, followed by EGF treatment for 20 min. All cells were subjected to SDEM, and the I- and S-fractions were analyzed by immunoblot. p-EGFR, EGFR phosphorylated on Y1173 detected with a phospho-specific Ab. B, cell viability measured by trypan blue exclusion 1 h after treatment of cells with filipin.

EGFR phosphorylation recovered to the levels observed in cells not treated with filipin (Fig. 2), suggesting that EGFR signaling is mediated by lipid rafts.

To determine whether EGFR/lipid raft signaling can mediate a prosurvival effect in LNCaP cells, EGFR-dependent cell survival was evaluated. Previous studies have shown that cell survival in LNCaP cells is enhanced by EGFR activation when apoptosis is stimulated by PI3K inhibitors (14). Consistent with published data, the PI3K inhibitor LY294002 triggered apoptosis in LNCaP cells, and the apoptotic effect of this drug was reversed by treatment with EGF (Fig. 3). Surprisingly, filipin alone stimulated apoptosis to a similar extent as the PI3K inhibitor, suggesting that disrupting the cholesterol-rich rafts not only interferes with EGFR signaling but also inhibits a critical cell survival pathway that is operating constitutively. The filipin effect was not attributable to membrane permeabilization because, at the doses used in these experiments, 100% of the cells excluded trypan blue 1 h after treatment (Fig. 2). Under conditions in which cells were treated with filipin, EGF pretreatment did not protect the cells from the apoptotic stimulus. In contrast, when membrane cholesterol was replenished after filipin treatment, the protective effect of EGF was again observed.

The Akt1 serine-threonine kinase is a prominent prosurvival signaling protein that is both downstream from EGFR activation and constitutively up-regulated in LNCaP cells. Given the strong inhibitory effects of filipin on EGFR activation and its ability to stimulate apoptosis, we hypothesized that filipin exerts a negative effect on Akt1 activity. Akt1 phosphorylation increased in response to 20 ng/ml EGF (Fig. 4A), consistent with a previous report from our group (14). EGF-induced Akt1 phosphorylation was substantially reduced in cells treated with 2  $\mu$ g/ml filipin. Significantly, both constitutive and EGF-induced EGFR and Akt1 phosphorylation were decreased with filipin treatment in a dose- and time-dependent fashion (Fig. 4, A, C,



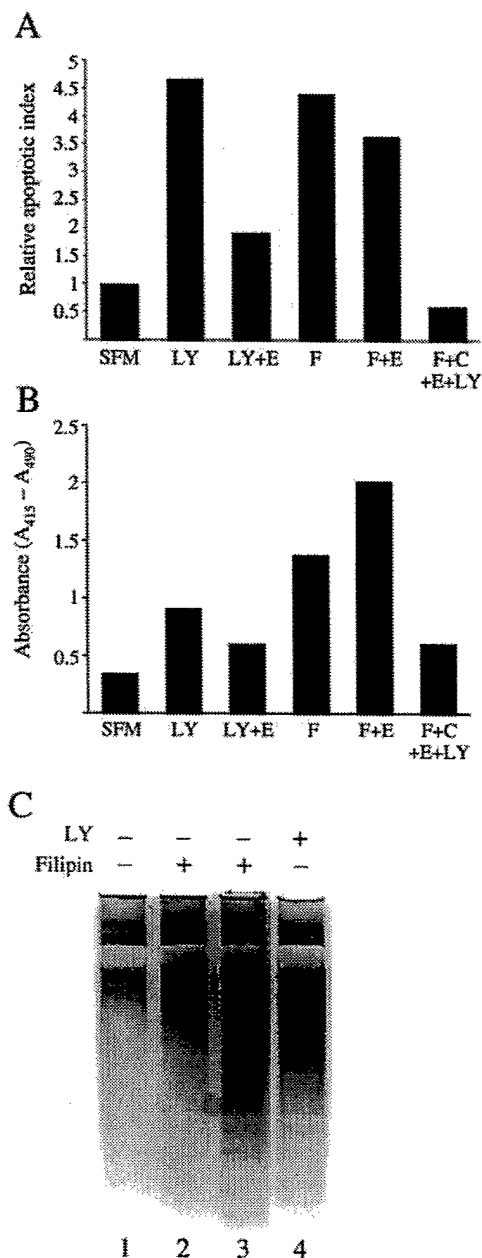


Fig. 3. Membrane cholesterol in lipid rafts mediates EGFR- and PI3K-dependent survival signals. **A**, apoptosis was quantified by TUNEL in combination with flow cytometry. LNCaP cells were seeded 24 h before treatments in six-well plates at a density of  $2-4 \times 10^5$ /well. Conditions shown are: 20 h in serum-free medium (SFM); 20  $\mu$ M LY294002 (LY); 20  $\mu$ M LY294002 with 10 ng/ml EGF treatment (LY+E); 2  $\mu$ M/ml filipin for 1 h (F); filipin treatment as (F) with 10 ng/ml EGF for 20 h (F+E); treatment with 2  $\mu$ M/ml filipin and cyclodextrin/cholesterol complexes (C) for 1 h, followed by incubation with serum-free medium containing 20  $\mu$ M LY294002 and 10 ng/ml EGF for 20 h (F+C+LY+E). **B**, quantitative measurement of DNA fragmentation was performed with a cell death detection ELISA kit. Cells were treated as in **A**. The results shown in **B** are averages of two independent experiments. All SDs are <10% of the values shown. **C**, assessment of oligonucleosomal DNA fragmentation, as an indicator of filipin-induced apoptosis. LNCaP cells were pretreated without (Lane 1) or with 0.5, 1  $\mu$ M/ml filipin (Lanes 2 and 3) for 1 h. Cells were then cultured in fresh serum-free medium for another 20 h. LY294002 (20  $\mu$ M, Lane 4) treatment time was 20 h in serum-free medium. After extraction, precipitated DNAs were separated in 1.8% agarose gels and visualized by digital capture of the ethidium bromide staining pattern.

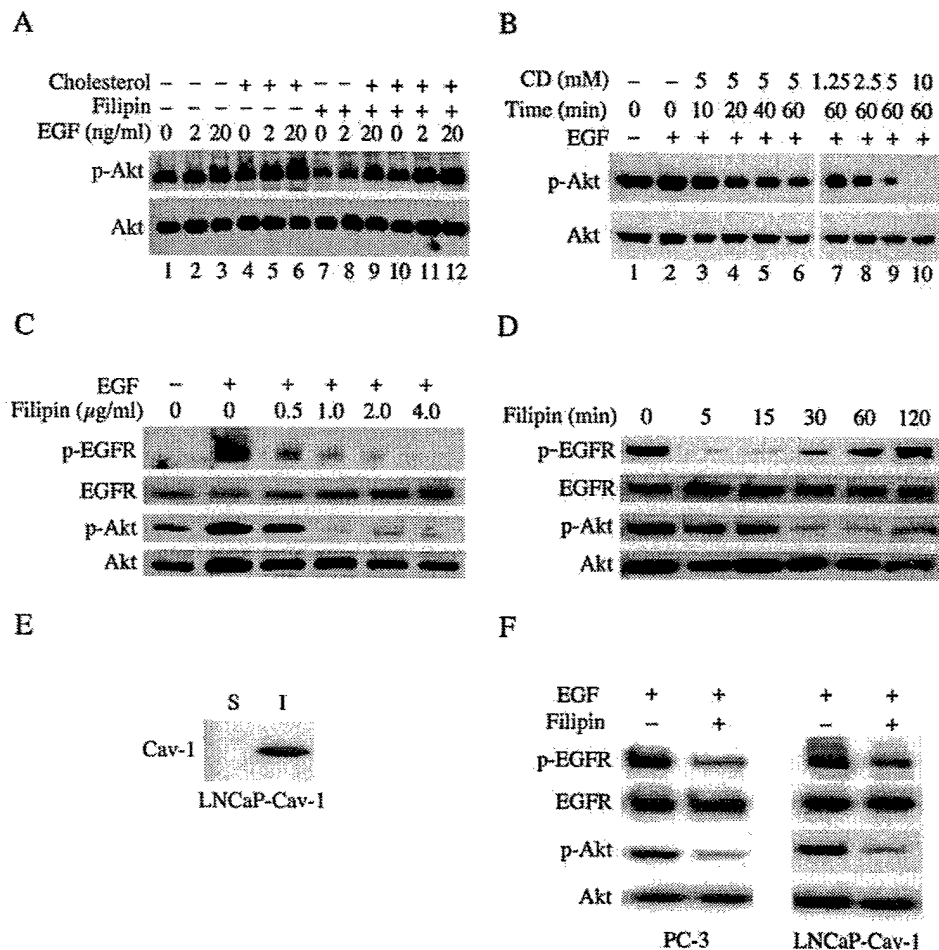
and **D**). Therefore, although the EGFR axis was affected by this agent, the inhibitory effect of filipin on Akt1 phosphorylation was not dependent on stimulation with exogenous EGF, a result that is consistent with the apoptosis results shown in Fig. 3. Similar inhibitory

effects on Akt1 phosphorylation were also demonstrated after membrane cholesterol was depleted with cyclodextrin, another cholesterol-binding drug (Fig. 4B). After cell membranes were loaded with cholesterol to reconstitute the raft microdomains, constitutive and EGF-induced levels of phosphorylated Akt1 were restored in filipin-treated (Fig. 4A, compare Lanes 7–9 versus Lanes 10–12) and cyclodextrin-treated (not shown) cells. Cholesterol repletion alone (in the absence of EGF and/or filipin) did not induce changes in Akt1 phosphorylation, and total Akt1 levels did not change with any of the treatments described above. These experiments were repeated with another EGFR ligand, HB-EGF, and similar results were obtained (data not shown). These findings indicate that EGFR→Akt1 signaling, as well as constitutive signaling through Akt1, appear to be under partial control of a cholesterol-rich membrane domain in LNCaP cells. This result is intriguing because Akt1 is believed to be stably activated in cells (such as LNCaP) that do not express a functional PTEN phosphatase. Our findings here indicate that despite stable up-regulation of Akt1 activity in a PTEN-null background, this kinase still remains partly dependent on an upstream, cholesterol-dependent signaling mechanism.

Caveolin-negative LNCaP cells stably transfected with caveolin-1 were used to determine whether expression of this protein, which is functionally involved in structural organization and cell signaling through caveolar lipid rafts (13), alters the apparent regulatory role of membrane cholesterol demonstrated above. Transfected caveolin-1 partitioned into the I-fraction as anticipated (Fig. 4E). Filipin treatment suppressed ligand-induced EGFR and Akt1 phosphorylation in both caveolin-transfected LNCaP cells and in PC-3 cells (Fig. 4F). In PC-3 cells, which express high levels of endogenous caveolin (Fig. 1), the levels of total and phosphorylated EGFR and Akt1 were very similar to those seen in the wild-type LNCaP cells, which do not express caveolins. In addition, the inhibitory effect of filipin treatment was also indistinguishable between the caveolin-expressing cells and unmodified LNCaP cells. These findings indicate that caveolin-1 does not regulate, nor does the presence of caveolin in the I-fraction alter, the effects of lipid raft disruption on the EGFR→Akt pathway in these cell types.

Our findings are the first to identify an important role for membrane cholesterol in the transmission of cell survival signals through the EGFR→PI3K/Akt1 pathway. We show that, in LNCaP cells, cholesterol-rich lipid rafts appear to be important for constitutive signaling through the Akt1 kinase, which is up-regulated in this cell line because the PTEN phosphatase is inactive. Because activation of PI3K/Akt signaling is thought to be an important, clinically relevant attenuator of apoptotic signals in PCa and other human malignancies, our current study suggests that, despite the absence of PTEN, signaling through Akt1 is still subject to down-regulation via alteration of membrane composition. This result suggests the possibility that targeting membrane cholesterol is a rational means for therapeutically down-regulating this pathway. This hypothesis is supported by published evidence demonstrating that polyene macrolide sterol-binding compounds, including filipin, significantly reduced prostate glandular hyperplasia in dogs by up to 75% with no toxicity (30). This effect, which was highly tissue specific (possibly because the prostate accumulates high levels of cholesterol), may be the result of disruption of cholesterol-mediated cell survival mechanisms. The presence or absence of caveolin-1 did not detectably alter the dependence of EGFR and Akt phosphorylation on cholesterol on intact lipid rafts, suggesting that the EGFR/PI3K/Akt cell survival axis is not dependent on the expression of caveolin proteins, and further, that down-regulation of this mechanism can be accomplished in caveolin-positive cells. Caveolin-1 expression has been linked recently to aggressive PCa (9, 31). Our studies provide a new mechanistic framework for the explo-

Fig. 4. Membrane cholesterol, not caveolin-1, is a key mediator of EGFR→Akt1 signaling. LNCaP cells were used for all experiments shown. **A**, before challenge with EGF (2 or 20 ng/ml), some groups of cells were treated with filipin (2  $\mu$ g/ml; Lanes 7–12) for 1 h. Other cells (Lanes 4–6 and 10–12) were subsequently incubated with cyclodextrin/cholesterol complexes (Cholesterol) for 1 h to replenish cholesterol. In this and subsequent panels, lysates were isolated following the various treatments and processed for immunoblot analysis. **p-Akt**, a phosphorylated form of Akt1 detected with a phospho-specific Ab (Ser-473). **B**, cells were serum starved overnight and then pretreated with 5 mM cyclodextrin for the indicated times (Lanes 1–6), or treated for 1 h with various concentrations of cyclodextrin (Lanes 7–10) and then treated immediately afterward with vehicle or with 5 ng/ml EGF. **C**, cells were either mock-treated or treated with various doses of filipin as indicated for 1 h, followed by 5 ng/ml EGF treatment. **D**, serum-containing medium was removed, and cells were treated immediately with 2  $\mu$ g/ml filipin alone under serum-free conditions for the times shown. No change in EGFR or Akt1 phosphorylation was observed when serum was removed, and cells were not treated with filipin (not shown). **E**, caveolin-1-transfected LNCaP cells (LNCaP-Cav-1) were fractionated into I- and S-fractions as above, and immunoblotting was performed with anti-caveolin-1 mAb. **F**, LNCaP-Cav-1 cells and PC-3 cells were mock-treated or treated with 2  $\mu$ g/ml filipin for 1 h at 37°C. Before the collection of cell lysates and immunoblot analysis, cells were stimulated with 5 ng/ml EGF for 20 min at 37°C.



ration of a role for cholesterol as a mediator of PCa development and progression.

Observations of cholesterol and other lipids accumulating in solid tumors, including PCa, have a long history (3). In this regard, it is interesting to point out that circulating cholesterol is a major source of plasma membrane cholesterol as a result of cellular absorption of lipoprotein from serum, and furthermore, that membrane levels of cholesterol can be substantially modified by diet (32). Rates of PCa progression are significantly affected by exogenous factors, including a Western diet and consumption of red meat and/or dietary fat (2). These observations may be related to the present findings that cholesterol-rich membrane microdomains regulate a survival function in human PCa cells. Furthermore, our observations may help provide a mechanistic link between cholesterol-rich diets and certain other diseases in which high-cholesterol, high-fat diets have been historically and epidemiologically associated.

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## References

- Kolonel, L. N., Nomura, A. M., and Cooney, R. V. Dietary fat and prostate cancer: current status. *J. Natl. Cancer Inst.*, 91: 414–428, 1999.
- Michaud, D. S., Augustsson, K., Rimm, E. B., Stampfer, M. J., Willet, W. C., and Giovannucci, E. A prospective study on intake of animal products and risk of prostate cancer. *Cancer Causes Control*, 12: 557–567, 2001.
- White, R. M. On the occurrence of crystals in tumours. *J. Pathol. Bacteriol.*, 13: 3–10, 1909.

- Jowett, M. The phosphatide and cholesterol contents of normal malignant human tissues. *Biochem. J.*, 25: 1991–1998, 1931.
- Yasuda, M. Lipid metabolism of tumors. *Proc. Soc. Exp. Biol. Med.*, 27: 944–945, 1930.
- Swyer, G. I. M. The cholesterol content of normal and enlarged prostates. *Cancer Res.*, 2: 372–375, 1942.
- Avecedo, H. F. E. A. Urinary cholesterol. V. Its excretion in men with testicular and prostatic neoplasms. *Cancer (Phila.)*, 32: 196–205, 1973.
- Parton, R. G. Caveolae and caveolins. *Curr. Opin. Cell Biol.*, 8: 542–548, 1996.
- Yang, G., Truong, L. D., Timme, T. L., Ren, C., Wheeler, T. M., Park, S. H., Nasu, Y., Bangma, C. H., Kattan, M. W., Scardino, P. T., and Thompson, T. C. Elevated expression of caveolin is associated with prostate and breast cancer. *Clin. Cancer Res.*, 4: 1873–1880, 1998.
- Yang, G., Addai, J., Ittmann, M., Wheeler, T. M., and Thompson, T. C. Elevated caveolin-1 levels in African-American versus white-American prostate cancer. *Clin. Cancer Res.*, 6: 3430–3433, 2000.
- Tahir, S. A., Yang, G., Ebara, S., Timme, T. L., Satoh, T., Li, L., Goltsov, A., Ittmann, M., Morrisett, J. D., and Thompson, T. C. Secreted caveolin-1 stimulates cell survival/clonal growth and contributes to metastasis in androgen-insensitive prostate cancer. *Cancer Res.*, 61: 3882–3885, 2001.
- Sargiacomo, M., Sudol, M., Tang, Z., and Lisanti, M. P. Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. *J. Cell Biol.*, 122: 789–807, 1993.
- Wary, K. K., Mariotti, A., Zurzolo, C., and Giancotti, F. G. A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. *Cell*, 94: 625–634, 1998.
- Lin, J., Adam, R. M., Santiestevan, E., and Freeman, M. R. The phosphatidylinositol 3'-kinase pathway is a dominant growth factor-activated cell survival pathway in LNCaP human prostate carcinoma cells. *Cancer Res.*, 59: 2891–2897, 1999.
- Cairns, P., Okami, K., Halachmi, S., Halachmi, N., Esteller, M., Herman, J. G., Jen, J., Isaacs, W. B., Bova, G. S., and Sidransky, D. Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. *Cancer Res.*, 57: 4997–5000, 1997.
- McMenamin, M. E., Soung, P., Perera, S., Kaplan, I., Loda, M., and Sellers, W. R. Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. *Cancer Res.*, 59: 4291–4296, 1999.
- Graff, J. R. E. A. Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27Kip1 expression. *J. Biol. Chem.*, 275: 24500–24505, 2000.

18. Lu, M. L., Schneider, M. C., Zheng, Y., Zhang, X., and Richie, J. P. Caveolin-1 interacts with androgen receptor. A positive modulator of androgen receptor mediated transactivation. *J. Biol. Chem.*, 276: 13442–13451, 2001.
19. Solomon, K. R., Mallory, M. A., and Finberg, R. W. Determination of the non-ionic detergent insolubility and phosphoprotein associations of glycosylphosphatidylinositol-anchored proteins expressed on T cells. *Biochem. J.*, 334: 325–333, 1998.
20. Li, S., Couet, J., and Lisanti, M. P. Src tyrosine kinases, G $\alpha$  subunits, and H-Ras share a common membrane-anchored scaffolding protein, caveolin. Caveolin binding negatively regulates the auto-activation of Src tyrosine kinases. *J. Biol. Chem.*, 271: 29182–29190, 1996.
21. Li, L., Yang, G., Ebara, S., Satoh, T., Nasu, Y., Timme, T. L., Ren, C., Wang, J., Tahir, S. A., and Thompson, T. C. Caveolin-1 mediates testosterone-stimulated survival/clonal growth and promotes metastatic activities in prostate cancer cells. *Cancer Res.*, 61: 4386–4392, 2001.
22. Okano, J., Gaslightwala, I., Birnbaum, M. J., Rustgi, A. K., and Nakagawa, H. Akt/protein kinase B isoforms are differentially regulated by epidermal growth factor stimulation. *J. Biol. Chem.*, 275: 30934–30942, 2000.
23. Ushio-Fukai, M., Hilenski, L., Santanam, N., Becker, P. L., Ma, Y., Griendling, K. K., and Alexander, R. W. Cholesterol depletion inhibits epidermal growth factor receptor transactivation by angiotensin II in vascular smooth muscle cells. Role of cholesterol-rich microdomains and focal adhesions in angiotensin II signaling. *J. Biol. Chem.*, 276: 48269–48275, 2001.
24. Bolard, J. How do the polyene macrolide antibiotics affect the cellular membrane properties? *Biochim. Biophys. Acta*, 864: 257–304, 1986.
25. Montesano, R., Vassalli, P., and Orci, L. Structural heterogeneity of endocytic membranes in macrophages as revealed by the cholesterol probe, filipin. *J. Cell Sci.*, 51: 95–107, 1981.
26. Santos, N. C., Ter-Ovanesyan, E., Zasadzinski, J. A., Prieto, M., and Castanho, M. A. Filipin-induced lesions in planar phospholipid bilayers imaged by atomic force microscopy. *Biophys. J.*, 75: 1869–1873, 1998.
27. Rothberg, K. G., Ying, Y. S., Kamen, B. A., and Anderson, R. G. Cholesterol controls the clustering of the glycosylphospholipid-anchored membrane receptor for 5-methyl-tetrahydrofolate. *J. Cell Biol.*, 111: 2931–2938, 1990.
28. Schnitzer, J. E., Oh, P., Pinney, E., and Allard, J. Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J. Cell Biol.*, 127: 1217–1232, 1994.
29. Liu, J., Oh, P., Horner, T., Rogers, R. A., and Schnitzer, J. E. Organized endothelial cell surface signal transduction in caveolae distinct from glycosylphosphatidylinositol-anchored protein microdomains. *J. Biol. Chem.*, 272: 7211–7222, 1997.
30. Gordon, H. W., and Schaffner, C. P. The effect of polyene macrolides on the prostate gland and canine prostatic hyperplasia. *Proc. Natl. Acad. Sci. USA*, 60: 1201–1208, 1968.
31. Yang, G., Truong, L. D., Wheeler, T. M., and Thompson, T. C. Caveolin-1 expression in clinically confined human prostate cancer: a novel prognostic marker. *Cancer Res.*, 59: 5719–5723, 1999.
32. Gray, D. F., Hansen, P. S., Doohan, M. M., Hool, L. C., and Rasmussen, H. H. Dietary cholesterol affects Na<sup>+</sup>-K<sup>+</sup> pump function in rabbit cardiac myocytes. *Am. J. Physiol.*, 272: H1680–H1689, 1997.